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Biological responses of two marine organisms of ecological relevance to on-going ocean acidification and global warming☆

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ABSTRACT

Recently, there has been a growing concern that climate change may rapidly and extensively alter global ecosystems with unknown consequences for terrestrial and aquatic life. While considerable emphasis has been placed on terrestrial ecology consequences, aquatic environments have received relatively little attention. Limited knowledge is available on the biological effects of increments of seawater temperature and pH decrements on key ecological species, i.e., primary producers and/or organisms representative of the basis of the trophic web. In the present study, we addressed the biological effects of global warming and ocean acidification on two model organisms, the microbenthic marine ciliate *Euplotes crassus* and the green alga *Dunaliella tertiocleta* using a suite of high level ecological endpoint tests and sub-lethal stress measures. Organisms were exposed to combinations of pH and temperature (TR1: $7.9_{[pH]}$, 25.5 °C and TR2: $7.8_{[pH]}$, 27,0 °C) simulating two possible environmental scenarios predicted to occur in the habitats of the selected species before the end of this century. The outcomes of the present study showed that the tested scenarios did not induce a significant increment of mortality on protozoa. Under the most severe exposure conditions, sub-lethal stress indices show that pH homeostatic mechanisms have energetic costs that divert energy from essential cellular processes and functions. The marine protozoan exhibited significant impairment of the lysosomal compartment and early signs of oxidative stress under these conditions. Similarly, significant impairment of photosynthetic efficiency and an increment in lipid peroxidation were observed in the autotroph model organism held under the most extreme exposure condition tested.

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1. Introduction

As a consequence of the industrial revolution, the atmospheric concentration of carbon dioxide (CO_2) is constantly increasing (Feely et al., 2004). A significant fraction of anthropogenic CO_2 is absorbed in the oceans (Canadell et al., 2007; Sabine et al., 2004), causing changes in the chemical and physical state of this gas and alterations in a wide range of abiotic and biotic processes. Any increase in the level of atmospheric carbon dioxide shifts the carbon dioxide–carbonate equilibrium in water in the acidic direction. Consequently, the oceans are acidified (for reviews on ocean acidification, see Fabry et al., 2009; Feely et al., 2009). Observations and models indicate that the average pH of the surface ocean has declined from 8.2 by 0.1 units since pre-industrial times as a result of CO_2 emissions and is projected to be approximately pH 7.8 by the end of this century (Raven et al., 2005). The ecological impact of ocean acidification resulting from the uptake of anthropogenic emissions of carbon dioxide is a relatively new concern for the scientific community and has attracted significant and increasing interest. However, while considerable emphasis has been placed on terrestrial ecology consequences, including forest repositories of

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carbon dioxide (Helliwell et al., 2014; Malcolm et al., 2014), the effects on marine environments have received little attention. Studies have primarily addressed reductions in coral calcification (Kato et al., 2014; Movilla et al., 2012), the survival, growth and shell integrity of crustaceans and other invertebrates (Bressan et al., 2014; Punt et al., 2014; Clements and Hunt, 2014) and the induced effects on fitness and/or unpaired development of fishes of economic value worldwide (Bromhead et al., 2015). However, little is known regarding the biological effects of seawater pH decrements on key ecological species, i.e., primary producers and/or organisms representative of the basis of the trophic web (Bermudez et al., 2015; Feng et al., 2017; Liu et al., 2017; Riebesell et al., 2017). Nevertheless, potential negative effects on these organisms may lead to a loss of biodiversity and irreversible changes in ecological structure, functioning and services (Mostofa, 2016; Sweetman and Chapman, 2015; Williamson et al., 2017). Indeed, autotrophic phytoplankton is an important carbon dioxide consumer group (Falkowski, 2012; Field et al., 1998). Since photosynthesis is the major consumer of carbon dioxide, any induced changes of phytoplankton abundance significantly affect the overall carbon dioxide balance. However, the current understanding of the potential effects of acidification on carbon dioxide removal from the atmosphere by phytoplankton is poor (Cermeño et al., 2008).

As well as the potential threat from ocean acidification, seawater temperature increases due to climate change may lead to increases in the metabolic rate of organisms. This aspect further increases the mismatch between primary production and respiration (Hoegh-Guldberg, 2010). The combination of these affects has the potential to generate crucial consequences for the microorganism community, thereby leading to changes in biogeochemical cycles and energy mediation between trophic levels and is therefore worthy of further investigation.

With climate change and ocean acidification becoming major concerns, additional mechanistic studies in aquatic research are needed to address potential interactions between organisms and observed or predicted changes in environmental conditions. These studies are required to increase the general awareness of how environmental parameters in aquatic environments may affect the precious balance of aquatic food webs with far-reaching consequences to the Earth's carbon dioxide level, marine conditions, and aquatic ecosystems. In this context, we assessed the biological effects of combined seawater increments of temperature and decrements of pH on two model organisms.

The green alga *Dunaliella tertiocleta* was selected as a representative species of marine primary producers, while the marine ciliate *Euplotes crassus* was selected for its key role as a heterotroph species in microbenthic environments. Growth rate, PSII efficiency and oxidative stress were investigated in the autotroph *D. tertiocleta*. In the eukaryote *E. crassus,* measurements of cell viability and replication rate were combined with the stress biomarkers, lysosomal membrane stability, lipofuscin accumulation and phagocytosis rate (Moore et al., 2006).

2. Materials and methods

2.1. Cell culture

Cells of *Euplotes crassus* were isolated from samples collected in the Tyrrhenian Sea (Italy) and grown under controlled laboratory conditions. Organisms were cultured in standardised artificial seawater (ASTM D1141-98; 34‰, pH = 8.1, 24 ± 0.5 °C) in an incubator chamber FTC 90 (VELP Scientifica Spa, Milan, Italy). Cultures were grown under dark conditions with an oxygen concentration ≥8.5 mg/L and fed weekly with *D. tertiocleta* (900 cells/mL). The analyses on ciliated protozoa were then performed during the logarithmic growth phase.

D. tertiocleta cells were isolated from water samples from the same geographical area and subsequently identified according to Preisig (1992). The obtained axenic algal culture was grown in ASTM artificial seawater (ASW, ASTM, 2013) enriched with a sterilised liquid nutrient medium according to Lain (1991). Cultures were renewed weekly by inoculating 1 mL of algae culture into 250 mL of freshly prepared growth medium. The flasks were maintained in a growth chamber (FTC 90E, VELP Scientifica, Italy) at 22.0 ± 0.5 °C. To assure optimal culture growth conditions, continuous uniform illumination with a quantum flux of 0.35 × 1020 photons/m2/s ± 20 per cent in the spectral range 400–700 nm was provided by cool white fluorescent lamps.

2.2. Experimental design

To simulate scenarios of the interconnected effects of climate change and ocean acidification experienced by aquatic organisms within the end of this century, two different combinations of temperature and pH were set up according to the projections of the climatological model developed under the European Project on OCean Acidification project (EU-EPOCA, Gattuso et al., 2011). The CO_2 calculation routine CO_2SYS was used to calculate the parameters of the seawater carbonate system and assist the design of ocean acidification perturbation experiments (Bellerby et al., 2005, 2012; Lavigne and Gattuso, 2011, Table 1). The pH scale was considered as the total scale (mol/kg-SW), and carbonic acid constants were considered according to Mehrbach et al. (1973) and refit according to Dickson and Millero (1987). KSO_4 was calculated according to Dickson (1981). The results of carbonate system simulation are provided as Supplementary Electronic Material (SEM).

Two different combinations of pH and temperature were tested within the experimental phase to simulate the predicted trend of ocean acidification and warming: $7.9_{[pH]}$ @ 25.5 °C (TR1) and $7.8_{[pH]}$ @ 27.0 °C (TR2). These parameters were selected according to the outcomes of the climatological model established to simulate average changes of oceanographic chemical-physical conditions of the north west Mediterranean basin within the next century. Control organisms were maintained at $8.1_{[pH]}$ and 24 °C, as conditions typically recorded in the sea surface temperature of the Tyrrhenian basin during the summer (Brunetti et al., 2014; Macias et al., 2013). All glass and plastic ware used within the experiments were cleaned prior to use by overnight soaking in 1 M HNO_3 followed by rinsing three times in MilliQ, dried in laminar flow hood and treated with UV light for 2 h.

Experiments were performed in an open unsterile system comprising a 6-L polyethylene tank filled with ASW, which was maintained in continuous agitation using two stirring bars (Fig. 1). An integrated pH/thermometer was remotely connected to open a line of analytical grade CO_2 using an electro-valve set up when the ASW pH exceeded the setup value (Fig. 1, NESA Srl, Italy). The pH/thermometer probe was calibrated prior to each test according to Bresnahan et al. (2014) and Dickson et al. (2007). The entire system was placed in an incubator provided with internal light 60 μE/m2/s (PAR) to perform tests with algae. System status, temperature and pH profiles were continuously recorded using internal data loggers to provide accurate data for QC/QA purposes. The entire data set is available as SEM. Test organisms were placed in modified 50-mL PP Falcon tubes (Corning, USA). Briefly, the conical bottom was removed and replaced with a PTFE membrane disc filter (Pall, USA) of two different pore sizes, to maintain confined protozoa (10 μm) or algae (0.1 μm). This design enabled the optimal medium circulation inside all tubes. Both ciliated protozoa and algae were previously acclimatised for 7 days to each testing pH and temperature combinations by first increasing the medium pH and temperature

Table 1
Routine CO_2SYS calculation: results of carbonate system simulation.

Salinity (‰)	t(oC) in	P (dbars) in	Total P (mmol/kgSW)	Total Alkalinity in (mmol/kgSW)	TCO2 in (mmol/kgSW)	pH in	fCO2 in (matm)	pCO2 in (matm)	HCO3 in (mmol/kgSW)	CO3 in (mmol/kgSW)	CO2 in (mmol/kgSW)	B Alk in (mmol/kgSW)	OH in (mmol/kgSW)	P Alk in (mmol/kgSW)	Revelle in	WCa in	WAr in	xCO2 in (dry at 1 atm) (ppm)
32	24,0	0	0,00	2720	2443	8,00	551	553	2211	215	16,3	72,6	5,2	0,0	11,2	5,3	3,5	569
32	25,5	0	0,00	2720	2487	7,90	720	722	2280	186	20,5	62,2	4,7	0,0	12,2	4,6	3,0	746
32	27,0	0	0,00	2720	2517	7,82	885	888	2324	168	24,3	55,3	4,5	0,0	12,9	4,1	2,7	920

at a constant gradient of 0.05 pH/day and 0.5 °C/day until reaching the final testing conditions (Fig. 1). These conditions were held for 10 days prior to obtaining endpoint measurements to address the effects of acclimation -like, rather than acute-like, responses on both autotroph and heterotroph organisms. Timing for acclimation was selected following an approach based on the generation times of the tested organisms. Expected generation time for *E. crassus* population in healthy conditions is of 8–9 h (Roth et al., 1985). All experiments were performed using a 12:12-h light:dark photoperiod. The ciliates were only fed during the acclimation and exposure period but not during tests execution. Temperature and pH were continuously recorded in all aquaria throughout the entire experimental period. At day 17 of the test phase (Fig. 1), nine modified Falcon tubes containing algae and twelve tubes containing protozoan sub aliquots as described below were placed in each of three 6-L tanks set up at C, TR1 and TR2 combinations of pH and temperature. For each pre-acclimatised condition (C, TR1, TR2) cells put into the modified Falcon tubes were collected from the same pre-test culture. Protozoan viability was assessed according to Gomiero et al. (2012) after transferring approximately 100 organisms from the exposure culture to each of three modified tubes placed in each of the above mentioned 6-L tanks. Similarly, ten individuals were transferred to three other tubes and maintained for 24 h prior to scoring the replication rate. The phagocytosis rate was scored on protozoan aliquots collected in a third set of Falcon tubes while a fourth set of three tubes was used for scoring lysosomal membrane stability and lipofuscin accumulation according to Gomiero et al. (2012). To assess the effects on algae, a batch of each culture pre-acclimatised to TR1, TR2 and control subpopulations were inoculated into freshly prepared medium maintained under testing conditions similar to those described above. The starting concentration in each of the testing Falcon tubes was 1.0×10^5 cells/mL. The growth inhibition test and the effects on photosystem II on marine algae were assessed after the 72-h incubation of freshly prepared cultures adjusted to the testing conditions in ASW medium.

3. Test on protozoa

3.1. Cell viability

Cell viability was assessed according to Gomiero et al. (2012). In each 5 ml well of a 24 well plate was placed a total of forty-sixty cells. Five wells were used for each exposure. The cell membrane impermeable DNA-binding probe SYBR Green™ (Sigma-Aldrich) was then added to each well after the incubation phase. The cells were observed at 200× magnification using an inverted microscope (Axiovert 100M, Zeiss, Germany) coupled with a FITC fluorescence filter setting and simultaneous light transmission. Dead cells were identified as showing a brightly fluorescent nucleus while live cells showed almost absent fluorescence. The images were captured, covering the entire well bottom of each sample, and the mortality rates were evaluated for each sample.

3.2. Replication rate

Changes in the mean fission rate were analysed after isolating five organisms into each well of a microplate according to Gomiero et al. (2012). Five wells were used for each exposure condition. The mean replication rate was estimated as follows:

$$n = \log 2\ n_x$$

where: n is the number of fissions after 24 h, n_x is the no. of organisms scored after 24 h and derived from a single isolated cell.

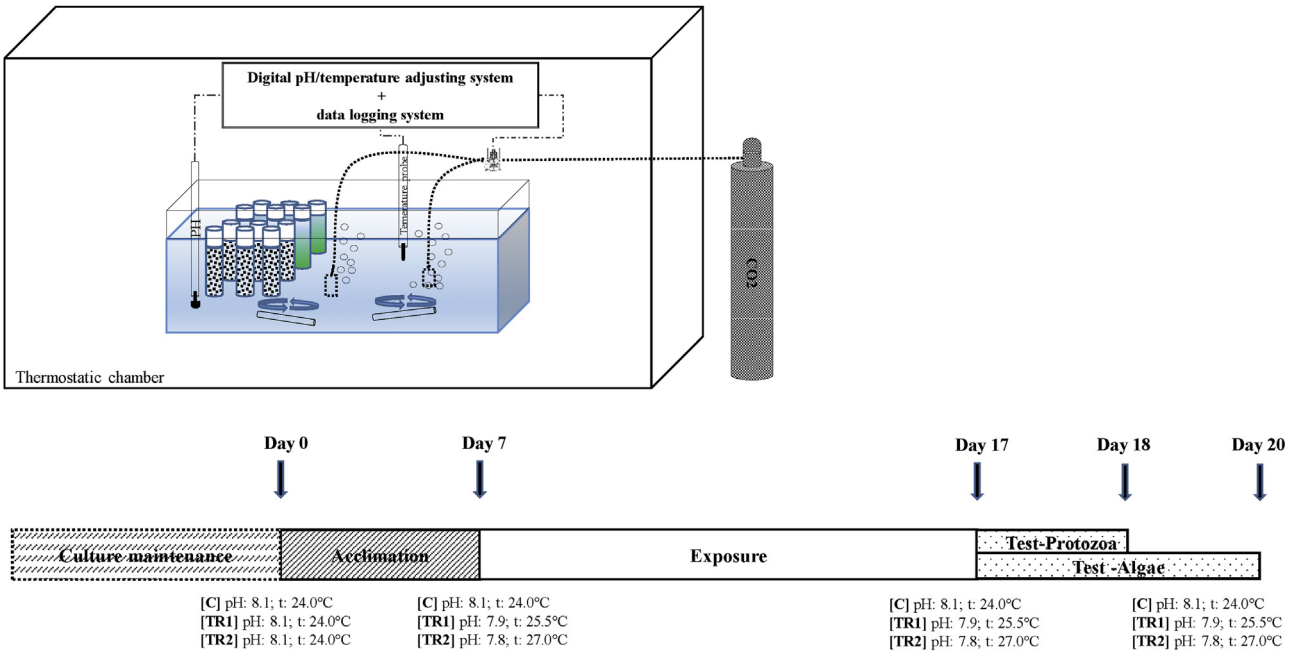

Fig. 1. Experimental design: overview of set acclimation/exposure conditions at specific points/frames within the experiments. [C] = Control, TR1 = I treatment conditions, TR2 = II treatment conditions.

3.3. Phagocytosis rate

To assess the phagocytosis rate, the cellular intake of fluorescent bioparticles of *E. coli* was quantified as described by Gomiero et al. (2012). After the exposure experiments, the protozoa were incubated with 100 μL of fluorescent bioparticles (*E. coli* K-12; Molecular Probes, US) in a 50 particles/μL concentration. Subsequently, the cells were fixed and approximately 50 individuals were placed on glass slides and a microscopic observation at 400× magnification was conducted on each of the slides. Fluorescence intensity was then expressed as a percentage of optical density with respect to control organisms.

3.4. Lysosomal membrane stability

The lysosomal membrane stability was estimated by testing the retention time of Neutral Red (NRRT) according to Gomiero and Viarengo (2014). The release of the NRRT dye within the lysosomes was monitored every 15 min up to 60 min and then after 90 and 120 min. Ten pictures were recorded for each replicate of every exposure condition. Approximately 40 individuals were scored and analysed using a semi-automatic image analysis system. The results were expressed as a percentage of optical density with respect to time 0. In the present study, NR retention time was evaluated on the whole-body area according to previously established criteria (Gomiero et al., 2012, 2013).

3.5. Lipid peroxidation

Lipid peroxidation was assessed by quantifying the intralysosomal accumulation of lipofuscins. These are pigmented lipid degradation products derived from peroxidative destruction of the cell's polyunsaturated lipid membranes (Terman and Brunk, 1998). An aliquot of protozoa was fixed in Baker's formol-calcium solution (15 min, 4 °C), pelleted and placed in Poly-L-Lysine-coated microscope slides. The cells were washed twice with ASW and subsequently incubated with Schmorl's solution (7 min, 25 °C). The staining reaction was terminated using a 1% acid acetic solution (1 min, 25 °C), and the samples were rinsed twice in Milli-Q water and mounted with glycerol gelatine. For each treatment, three microscope slides were prepared and the images of ten organisms per slide were obtained. The images were analysed using an image analysis system by selecting an optimal density index value for each image with the threshold option. Data were expressed as average pixels per area and cell.

4. Test on algae

4.1. Growth inhibition test

The growth inhibition of *D. tertiocleta* was estimated according to ASTM (2012). Aliquots of algae in the logarithmic phase were collected from a batch of enriched culture and inoculated into each tube to reach a starting concentration of approximately 1.0×10^5 cells/mL. After incubation for 72 h under the selected experimental conditions, three 1-mL aliquots from each Falcon tube were collected in 1.5 mL Eppendorf tubes and fixed with 10 μl of Lugol's solution. A volume of 10 μl of fixed cells were subsequently spotted onto disposable counting chamber slides and scored using a Countess Automated Cell Counter (Invitrogen, Italy). Growth curves were prepared from data obtained from sampling cultures at intervals, such as once per day, depending on the growth of the algae. Plots of number of cells against time (in days) were generated, and these curves were used to calculate the specific growth constant (U) as:

$$U = (\ln X_2 - \ln X_1)/(t_2 - t_1)$$

where X_1 and X_2 are densities at t_1 and t_2 and subsequently expressed as percentage (%) with respect to the control.

The QA/QC of automated counting procedures was assessed using 10-μm latex-based Countess® Test Beads (Invitrogen, Italy)

and is available as SEM. A preliminary experiment was also performed after counting cells using a Neubauer chamber.

4.2. Efficiency of photosystem II electron flow

A Dual-PAM 100 fluorometer (Walz, Germany) was used to assess the photosystem II (PSII) efficiency by measuring chlorophyll fluorescence. Prior to each measurement, the sample was dark-adapted for 10 min (Park et al., 2015). Pulses (0.5 s) of red light (5000 μmol m^{-2} s^{-1}) were used to determine the maximum fluorescence and the ratio between variable and maximal fluorescence $(Fm-F_0)/Fm = Fv/Fm$, a parameter used to calculate the maximum efficiency of PSII (when all reaction centres are open). The photochemical quenching (PQ) of chlorophyll fluorescence due to state transitions was determined by illuminating dark-adapted algae with red light (35 μmol m^{-2} s^{-1}, 15 min) and subsequently measuring the maximum fluorescence in state 2 (FM2). Next, state 1 was induced after adding far-red light (maximal light intensity corresponding to level 20 in the Dual-PAM setting, 15 min), and recording FM1. The qT was calculated as (FM1−FM2)/FM1 to measure the fluorescence yields of all cultures grown.

Non-Photochemical Quenching (NPQ) was calculated according to Maxwell and Johnson (2000) as

$$NPQ = (Fm-Fm')/Fm'$$

Quantum yield of non-regulated non-photochemical energy loss in the photosystem II [Y(NO)] is calculated as

$$Y(NO) = \frac{1}{NPQ + 1 + qL(\frac{Fm}{F0} - 1)}$$

The quantum yield of photochemical energy conversion in PS II [Y(II)] is calculated as

$$Y(II) = (Fm\prime - F)/Fm\prime = \Delta F/Fm\prime$$

where

Fm = Maximal fluorescence yield of dark-adapted sample with all PSII centres closed,
Fm' = Maximal fluorescence yield of illuminated sample with all PSII centres closed,
ΔF = Increase of fluorescence yield, F_m' − F, induced by a saturation pulse,
F0 = Minimal fluorescence yield of dark-adapted sample with all PS II centres open,
qL = Parameter estimating the fraction of open PSII centres based on the lake model.

4.3. Determination of total chlorophyll

Aliquots of 1 ml of microalgae culture were collected by an initial centrifugation at 5000×*g* for 10 min. The supernatant was discarded, the pellet resuspended and washed twice with ASW and further centrifuged. The obtained supernatant was discarded while the hard pellet resuspended in 1 ml of 100% methanol neutralised with magnesium carbonate. The crude extract was allowed to stand in a refrigerator in the dark at 6 °C for about 3 h. The precipitate was removed by centrifugation at 5000×*g* for 20 min and the supernatant collected for analysis. Spectrophotometric readings were performed by a Shimadzu Pharmaspec UV-1700 spectrophotometer using standard scanning settings and a 1 nm bandwidth and 1 nm sampling interval. Quartz cuvettes were used. The chlorophyll *a and b* and total chlorophyll estimations were performed according to Ritchie (2008):

4.4. Lipid peroxidation

Lipid peroxidation was measured using the cell permeable indicator C11-BODIPY 581/591. Each treatment and control was treated with 5 μM of C11-BODIPY581/591 in 1 mL of solution according to Oukarroum et al. (2012). The fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

4.5. Statistics and data analysis

An initial statistical analysis of the data based on a two-way ANOVA was conducted to discriminate differences among experimental conditions. Biological responses were compared with control organisms. The results were further analysed using the Student-Newman-Keuls multi-comparison test to statistically discriminate the sensitivity to the tested exposure conditions. Differences were considered to be significant when $p < .05$. Analyses were performed using Statistica 9.0 (Statsoft, USA).

5. Results

In Fig. 2A, the percentages of the survival rate of subpopulations of ciliate *E. crassus* exposed to the two tested combinations of pH and temperature are shown. Negligible rates of mortality (2–3%) were observed in the control organisms, excluding the likelihood of stress conditions in the ciliate cultures. However, no significant differences in survival rate were observed under all tested conditions with respect to the control organisms as rates decreased 6% and 9% at 7.9 $_{pH}$; 25.5 °C (TR1) and 7.8 $_{pH}$; 27,0 °C (TR2), respectively. However, TR2 conditions induced a moderate but significant decrement on the replication rate (−13%; $p < .05$) of protozoa with respect to control organisms, and no significant differences in the statistical analysis of the results under TR1 conditions were recorded (Fig. 2B). Moderate but progressive decrements of pH conditions and increments of seawater temperature induced few significant decrements of the lysosomal membrane stability in protozoa exposed to the most adverse conditions of TR2 (−13%) compared to control ($p < .05$), while no significant lysosomal compartment disruptions were observed within the TR1 exposure conditions (Fig. 2C). Similar to the results for the survival rate, no significant decrements of the phagocytosis rate were recorded, as percentages spanned over −9% and - 10% among the different exposure conditions compared to control organisms (Fig. 2D).

A similar pattern of response but with an opposite trend reflecting the mode of action of the adopted index was observed by assessing the intra lysosomal accumulation of lipofuscins, the end-products of the cell's lipids peroxidation. A few insignificant increments (+3%) were observed in protozoa exposed to the TR1 conditions compared to control organisms, while the TR2 conditions induced significant lipofuscin accumulation (+22%) in the lysosomes of treated protozoa ($p < .05$) (Fig. 2E).

The autotrophic biological model *D. tertiocleta* showed significant increments of the growth rates in both exposure conditions with respect to control ($p < .05$). Indeed, the 72 h incubation under the 7.9 $_{pH}$; 25.5 °C condition resulted in a significant increase of the growth rates in the treated green algae (+22%). Further increments of seawater temperature and decrements of pH values provoked an even higher positive (+36%, $p < .05$) stimulation of the growth rates in the same autotrophic organism (Fig. 3A). Control organisms reached an average growth rate (μ) of 0.85–1.25 d^{-1}, confirming the

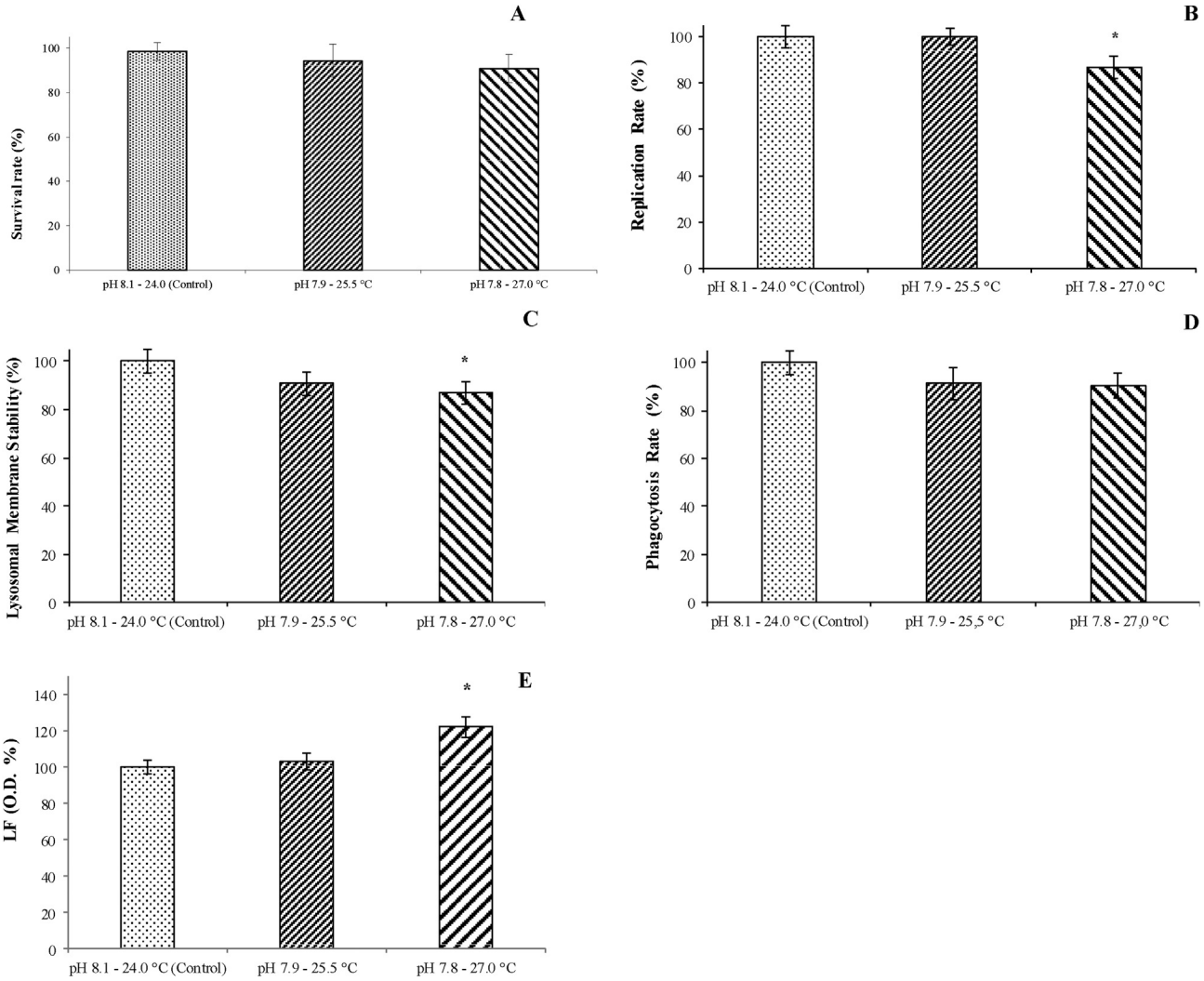

Fig. 2. – Biological responses of *E. crassus* submitted to TR1 [pH: 7,9; t: 25,5 °C] and TR2 [pH: 7,8; t: 27 °C] exposure conditions. Results of (A) survival rate (%), (B) replication rate (%), (C) lysosomal membrane stability test (%), (D) Phagocytosis rate (%) and (E) lipofuscin content (OD %). * = $p < 0,5$, Student–Newman–Keuls multi-comparison test. Error bars represent standard deviation; n = 5.

good health status of the algal batch used in the present study. On the other hand, the total chlorophyll concentration (Chl) decreased in organisms exposed to the two acidified conditions (TR1: 0.40; TR2: 0.35 fg/cell) with respect to control ones (0.64 fg/cell; $p < .05$, Fig. 3B). The distribution of chlorophyll *a and b* forms in particular has been detailed, them being the most widely distributed and actively contributing forms in the oxygenic photosynthesis of marine green microalgae. The chlorophyll quota to cell number normalisation showed a similar trend: after an initial similar level of approximately 0.35 fg $_{[Chl\ a+b]}$/cell in all sub aliquots, algae presented significantly lower values under the two acidic conditions at the end of the experiment (TR1: 0.18 fg $_{[Chl\ a+b]}$/cell; TR2: 0.14 fg $_{[Chl\ a+b]}$/cell) with respect to the controls (C: 0.33 fg [Chl a+b]/cell; Fig. 3C). The maximum potential quantum efficiency of Photosystem II (Fv/Fm), which provides an index of the efficiency of photosystem II photochemistry, declined from 0.68 ± 0.02 in control samples to 0.62 ± 0.02 under TR1 conditions and further decreased to 0.44 ± 0.03 under the more drastic conditions of TR2. The maximum value of Fv/Fm obtained under control conditions using the PAM technique was quantitatively similar to the maximum values observed in optimal conditions for *D. tertiocleta* cultures using the pump and probe fluorescence technique (Kolber et al., 1988).

The effect of increased CO_2 on the light harvesting-related parameters (antenna size and quantum yield) in *D. tertiocleta* was further investigated by examining the relative PSII electron transport rate (ETRII), and "primarily constitutive losses" Y(NO), corresponding to the sum of non-regulated heat dissipation and fluorescence emission (Fig. 4). While Y(II) and ETR(II) did not show significant variations at increased temperatures and acidity, the curves describing Y(NO) were clearly associated with the growing conditions: the higher the values of temperature and acidity, the higher the mean values of Y(NO) along the curve. Furthermore, the mean Y(NPQ) showed a similar response to the harshening conditions, but not as neatly proportional and clear a trend as in the case of Y(NO).

Algal cells treated under increasing acidic and temperature conditions indicated that only the most extreme tested pH and temperature combination TR2 induced a significant increment of lipid peroxidation with respect to control cells (+27%; $p < .05$; Fig. 5).

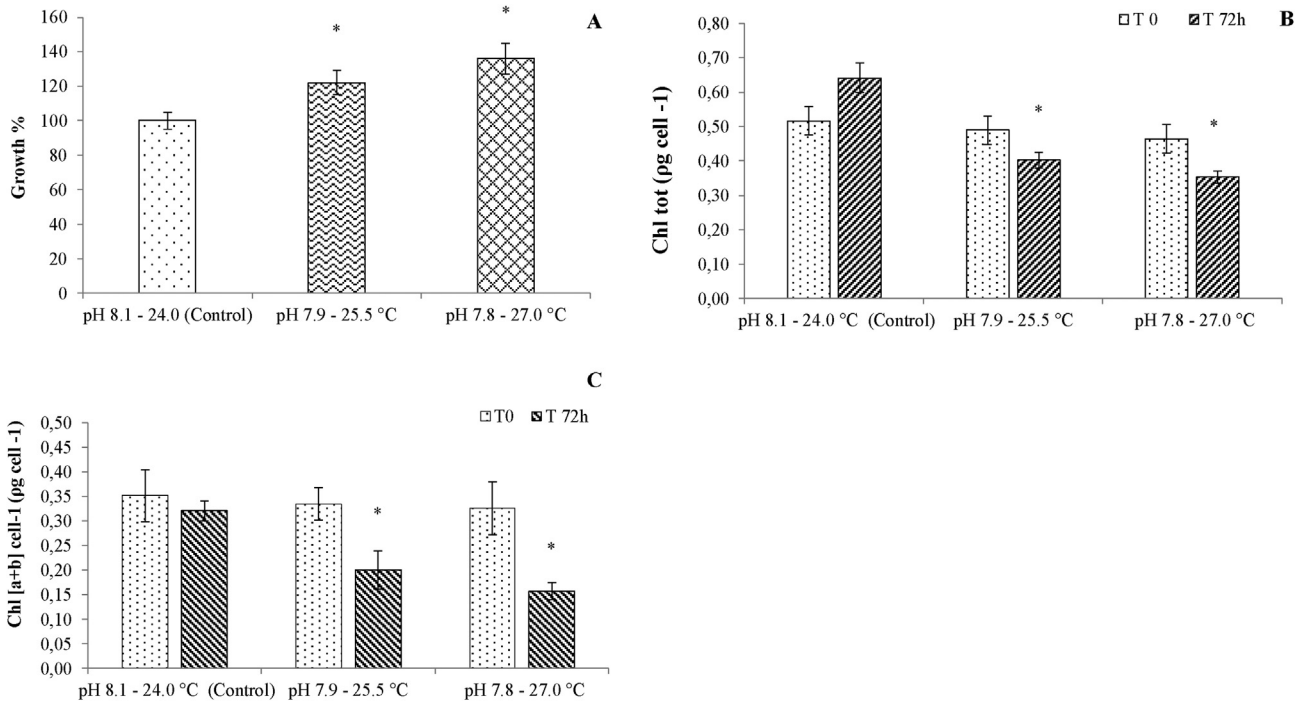

Fig. 3. - Biological responses of *D. tertiocleta* submitted to TR1 [pH: 7,9; t: 25,5 °C] and TR2 [pH: 7,8; t: 27 °C] exposure conditions. Results of (A) growth rate (%), (B) total chlorophyll content (μg/ml), (C) chlorophyll *a and b* content (ρg/cell); (D) PSII efficiency. * = $p < 0,5$, Student–Newman–Keuls multi-comparison test. Error bars represent standard deviation; n = 5.

6. Discussion

Increasing atmospheric carbon dioxide concentration is promoting global warming and acidifying oceans by changing the carbon dioxide-carbonate equilibrium in water (Feely et al., 2009). Several direct and indirect consequences have been forecast, ranging from disruption of nutrient cycles leading to consequent eutrophication phenomena, altering the availability of carbonate ions, a key building block for calcifiers of ecological relevance, i.e., corals (Gattuso and Hansson, 2011), and an increase in speciation and bioavailability of metals (Hoffmann et al., 2012; Lacoue-Labarthe et al., 2011). Such trends have become particularly evident in coastal areas (Cai et al., 2011; Pascal et al., 2010). The expected rise in temperature will drive an increase in the metabolic rate of organisms, primarily affecting localised oxygen concentrations in the aquatic environment but also leading to a cascade of indirect ecological consequences (Keeling et al., 2010). Autotrophs and heterotrophs may react quite differently to such changes (Gattuso et al., 2011; Mastrandrea et al., 2010). Reduction in seawater pH may induce changes in cytosolic pH, which can have a wide range of effects on heterotrophic organisms. This parameter plays a key role in many physiological processes, such as ion transport, sub-cellular organelle function, energy balance and protein synthesis (Rokitta et al., 2012; Steinberg, 2012). Furthermore, adverse effects on key species within food webs can affect ecosystem structures, leading to both a loss of biodiversity and alterations in ecological functioning (Brown et al., 2018; Nagelkerken and Connell, 2015; Nagelkerken et al., 2017).

In the present study, tests with high-level ecological relevance (i.e., mortality and replication rate estimation) together with informative sub-lethal endpoint assessments (lysosomal compartment alteration, predatory ability and oxidative stress) were performed on the ciliated protozoan (*Euplotes crassus*), selected as a representative of the marine microbenthic community. The ecological role of protozoa in the transfer of energy and matter of bacterial and algal production to successive trophic levels is crucial. Therefore, it is important to understand the possible consequences of ocean acidification and global warming induced changes on microbenthic community structure and functionality. The outcome of the present study showed that the scenarios tested did not result in a significant increase in mortality, suggesting the potential existence of an effective mechanism to maintain a relatively constant internal pH in protozoa. A number of ocean acidification studies have discussed mortality as a potential measure of organism sensitivity to acidified seawater (Gaylord et al., 2016; Gunderson et al., 2016; McGovern and Hall-Spencer, 2014), though results from the present study suggest that at least for protozoans, this particular endpoint would have a limited value. Trade-offs between maintenance activities, growth and/or reproduction might however be expected, therefore negative effects of ocean acidification may be detectable after analysing such trade-offs (Widdicombe and Spicer, 2008; Wood et al., 2008). Indeed, the results of sub-lethal stress indices in *Euplotes crassus* show homeostatic mechanisms at work and these will have energetic costs, which may have diverted energy from other essential processes, such as growth and replication (Bechmann et al., 2011; Sokolova et al., 2012). This effect was particularly evident for *E. crassus* exposed to the most stringent conditions, where significant reductions in replication rate were recorded.

Progressive proton enrichment in the intracellular environment arising from reduced seawater pH may interfere with normal cellular processes and kinetics leading to the disruption of some cellular functions. In this context, all biological membranes are enriched with lipid-soluble weak acids (Nikinmaa, 2013). These molecules accumulate in all biological membranes and act as protonophores shuffling protons across membranes. Under normal physiological conditions, the contribution of lipophilic weak acids is negligible. However, under progressive increments of free ions at

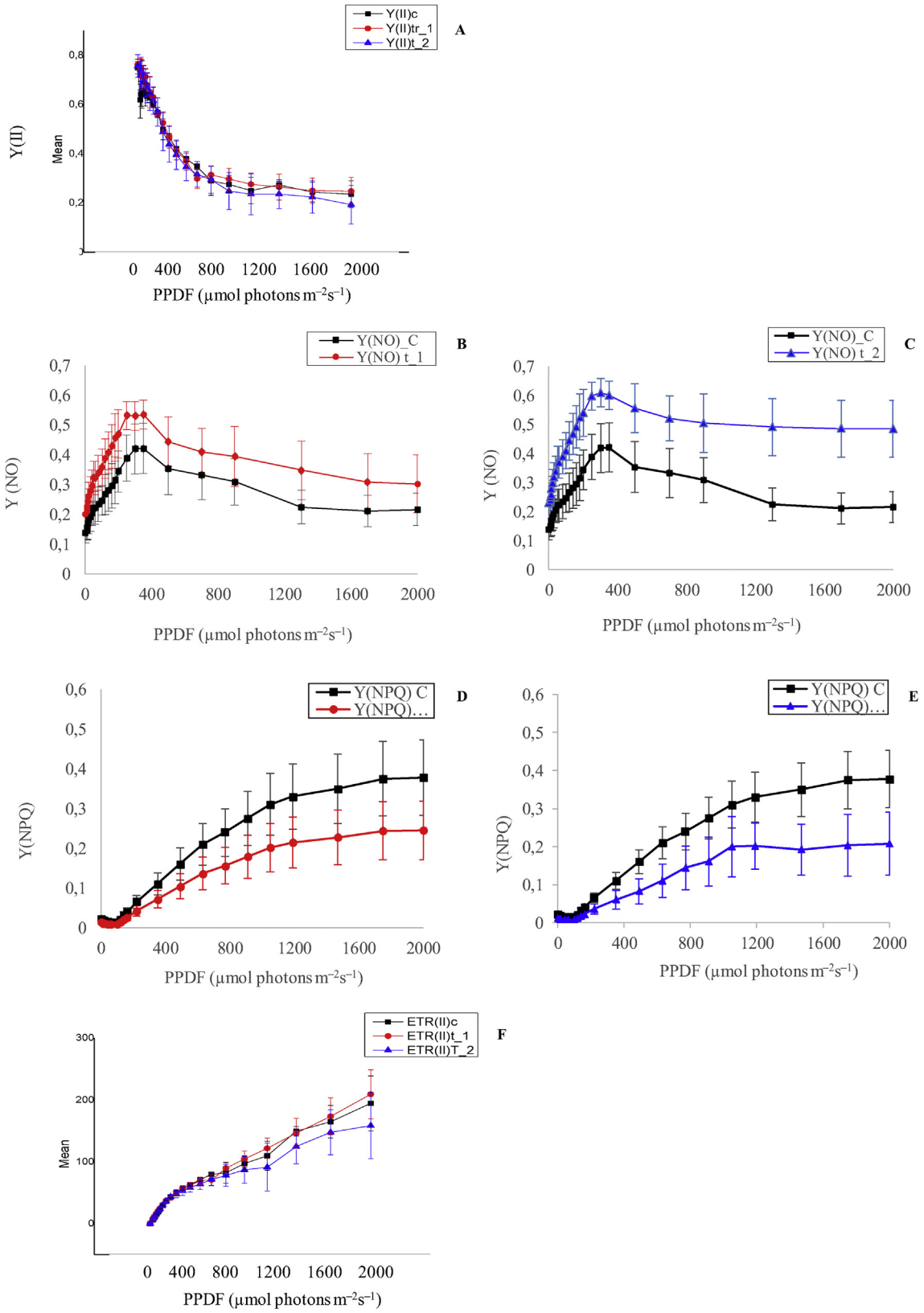

Fig. 4. - (A) The quantum yield of photochemical energy conversion in PS II [Y(II)], (B–C) the quantum yield of non-regulated non-photochemical energy loss in the photosystem II [Y(NO)], (D–E) Non-Photochemical Quenching fitness (NPQ) and (F) the relative Electron Transport Rate ETR(II). PPDF, Photosynthetic photon flux density (µmol photons $m^{-2}s^{-1}$). Error bars represent standard deviation; n = 10.

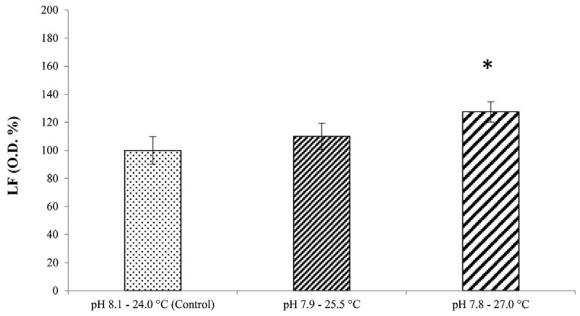

Fig. 5. Results of lipid peroxidation analysis in *D. tertiocleta* submitted to TR1 [pH: 7,9; t: 25,5 °C] and TR2 [pH: 7,8; t: 27 °C] exposure conditions. * = p < 0,5, Student–Newman–Keuls multi-comparison test. Error bars represent standard deviation; n = 10.

intracellular level, their competing role could be relevant, lowering the general energy production efficiency (Strobel et al., 2013).

Furthermore, a contemporary unpairing of intracellular proton levels together with an increment of the temperature may boost biochemical processes involving positive charged ions eventually leading to an increase of the cytosolic formation of free radicals (Tomanek et al., 2011). These molecules may lead to cellular oxidative stress in oxidising organelles, thus compromising their functioning, and the integrity of cell membranes. Indeed, significant intra lysosomal accumulation of lipofuscins and increments of lysosomal membrane permeability were observed in protozoa exposed to the most stringent exposure conditions within the present study. However, no significant increases in mortality rates or sub-lethal stress effects were observed in *E. crassus* exposed to variations of pH and temperature expected within 50 years from now. The limited biological responses observed in this study may reflect a combination of an efficient acid-base regulation system and a possible adaptation tendency of these unicellular organisms to the conditions tested (Hofmann and Todgham, 2010). Indeed, considering the short generation times, it is not unreasonable to hypothesise that these organisms may adapt in the timescale of ocean acidification, although with unknown consequences for higher trophic levels, which are unlikely to have adequate time to evolve (Blackford, 2010) Synergistic effects of climate-related variables suggest future physiological impairment in organisms with a complex biological organisation, i.e., those organised in tissues and organs (Rosa and Seibel, 2008). However, speculation about the potential climate change effects at higher levels of the ecological food web is beyond the scope of the present study.

Changes in climate may have indirect effects on the tolerance of marine organisms to multiple stressors by influencing the key mechanisms controlling the fate, behaviour and bioavailability of environmental pollutants (Gomiero et al., 2012; Macdonald et al., 2002; Noyes et al., 2009; Sweetman et al., 2005). This relationship deserves further investigation to estimate potential future effects on microbenthic organisms. Indeed, protozoa and bacteria live closely together interacting with sediments, an environmental compartment which often acts as a repository of organic and inorganic pollutants.

Shifts in the seawater carbon chemistry and temperature may also promote complex feedback-like responses in aquatic ecosystems. It has long been debated whether phytoplankton species are growth limited by current levels of carbon dioxide in aquatic systems and whether increments in the atmospheric CO_2 could stimulate growth (Riebesell et al., 1993). Indeed, while temperature and pH induced eutrophication processes tend to boost primary production consumption of carbon dioxide, overall respiration increases, leading to a decrease in oxygen level (Diaz and Rosenberg, 2008).

In the present study CO_2 induced decrease of pH stimulated the growth rate of algae; a relatively common response in the *Dunaliella* spp. (Gordillo et al., 1998, 2003). However, a combined decrease in pH and increase in temperature induced changes in the amount of cell pigment concentration, which progressively declined under the most extreme exposure condition tested. García-Sánchez et al. (1994) previously proposed a regulatory role for CO_2 on pigment composition and abundance. Qualitative pigment changes were also observed in cyanobacteria, where high levels of CO_2 induced both an increase in the number of phycobilisomes and a decrease in their size (Poza-Carrión et al., 2001). In the present study, the higher loads of CO_2 administered to decrease the pH levels may have promoted an increase in the number of PSII reaction centres of smaller size in the investigated *D. tertiocleta*. Indeed, the early findings of Sukenik et al. (1987) suggested that *Dunaliella* spp. modifies PSII excitation by altering the number of chlorophyll molecules bound to each protein in the light-harvesting pigment-protein complexes.

The decrease of the Fv/Fm ratio under the most extreme exposure condition tested indicates a loss of photosynthetic efficiency. This effect could partially reflect changes in the quenching characteristics of the excitons reaching the reaction centres. Lower PSII excitation pressure at low pH (high CO_2) would correspond to a higher demand for electrons exerted by C fixation that would act as a sink for excitons, reflected as a higher photosynthetic electron transport rate. This effect may lead to a decline of the excitation pressure, thus avoiding saturation. Low rETR could lead to the saturation observed in the PSII excitation pressure and consequently an increase in NPQ at a relatively high irradiance. Indeed, an increase of thermal dissipation of absorbed light was observed under the most severe conditions, corresponding to a lower capacity to harvest light. This effect is supported by the poor pigmentation and low Fv/Fm values observed under the same exposure conditions. These outcomes suggest that climate-driven impacts on keystone and foundation species may be particularly important, affecting important physiological processes. Early studies have addressed the response of open-ocean primary productivity to climate forcing at local to global scales.

These outcomes can be expressed through bottom-up impacts, such as decreases in water-column primary production and/or shifts towards smaller cells in planktonic communities and through top-down impacts that cascade down from the losses or gains of ecologically dominant consumers (Doney et al., 2012). Exposure to waters with low pH may disrupt the internal acid-base balance of organisms, which in turn can affect a wide variety of metabolic processes (Pörtner, 2010). Some organisms, particularly those with well-developed respiratory systems are more capable to maintain or restore internal acid-base balance, whereas less physiologically flexible taxa (e.g., protozoa) may be more vulnerable (Hendriks et al., 2015). However, in carbon-limited autotrophs, the increased CO_2 may promote photosynthesis, whereas for other organisms (particularly calcifying taxa), photosynthesis may be either reduced or unaffected (Kroeker et al., 2010).

7. Conclusions

The aquatic environment integrates the responses of organisms' physiologies and ecological interactions to changes in climate and CO_2. Consequently, the mechanisms linking changes in populations and communities to alterations in ecosystem-level properties, such as trophic structure, food-web dynamics, energy flow, and biogeochemical cycles, are diverse and have not been thoroughly elucidated, particularly on the ecosystem dynamics and feedback

processes (Hendriks et al., 2015). Disruptions of existing biological interactions lead to changes in community composition and biodiversity, and the loss of functionally prominent species (Doney et al., 2012). The contribution of the biological acclimatisation and the adaptation in modulating the rate of climate-driven marine ecosystem changes need to be further investigated Therefore it is crucial to establish a long-term ecosystem-based observational system revealing both which aspects of the marine ecosystem structure are most important for conferring resilience against climate stressors and which aspects are most amenable to management. Considering the circular economy and sustainable exploitation of the natural resources further research should determine whether climate change effects on aquatic ecosystem structure and function may affect the ability of society to rely on the flow of goods and services from the aquatic environment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.01.063.

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